THE POTENTIATION OF TETANUS TOXIN BY BROTH AND SERUM[1]

F. B. TRAUB, A. HOLLANDER, AND U. FRIEDEMANN

Department of Bacteriology, The Jewish Hospital of Brooklyn, Brooklyn, New York

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Years ago Ricketts and Kirk (1906), Marie (1914), Bronfenbrenner (1924), Coleman (1924), Wagner, Meyer, and Dozier (1925), Jensen (1926), and Zuger, Hollander, and Friedemann (1939) reported that botulinus and tetanus toxins are potentiated by a number of substances. Among these, nutrient broth and blood serum are of particular interest, the former because it is a constituent of all impurified tetanus toxins, the latter because some problems require the quantitative determination of tetanus toxin in blood. In the course of experiments with tetanus toxin it occurred to us that neglect of this potentiation effect might cause serious errors in the evaluation of the potency of tetanus toxin. It was felt, therefore, that concerning this phenomenon more information was needed than is available from the scanty data in the literature.

In the first place, this added information pertains to the quantitative aspects of the potentiation effect. The majority of the above-mentioned papers contain no data concerning the extent to which tetanus toxin is potentiated by undiluted broth or serum. What is more important, it is unknown how far these substances can be diluted and still give a marked potentiating effect. On the basis of the available information it is impossible, therefore, to predict whether the titration of tetanus toxins will be affected by the broth content of the limiting dilutions.

Experiments with a large number of tetanus toxins led to the unexpected observation that some toxins are very strongly potentiated by broth and serum but that other toxins are not potentiated at all. It was further observed that the potentiation phenomenon is very marked in some animal species but that it is absent in others.

A great deal of work was devoted to attempts at elucidating the mechanism of the potentiation phenomenon. Although, thus far, the results of these experiments were unsatisfactory, we are at least in position to decide the question whether broth and serum actually potentiate the toxin or whether, as has been claimed by some authors, they serve merely as buffers which prevent the deterioration of the toxin in saline. On the basis of our experimental data the titration of tetanus toxin under various conditions will be discussed.

METHODS AND MATERIAL

Toxins 1175 H, 641 B, 1346, and 1375 were placed at our disposal through the courtesy of the Lederle Laboratories, Inc. We are indebted for toxin 103 to the Laboratories of the New York City Health Department. Toxins G, 8, 3, 2, 22, H, 4, 7, 5, 15, 6, A, B, C, J, K, D, E, F, and L were prepared in our laboratory.

With few exceptions, all dilutions of toxin were made either in broth, serum,

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or saline. A separate pipette was used for each dilution. In experiments with toxin 1175 H we observed a very marked pipette error. In experiments with other toxins, however, it did not make any difference whether or not the pipettes were changed.

EXPERIMENTAL

Quantitative aspects of the potentiation phenomenon. Experiments with tetanus toxin 1556 in mice may serve as an illustration of the potentiating effect. Guinea pig serum, broth, and some constituents of broth were examined. As may be seen from table 1, guinea pig serum has the strongest potentiating effect (64 times); then follows broth (32 times). Difco peptone potentiates as strongly as

TABLE 1

Potentiation of tetanus toxin 1556 by guinea pig serum, nutrient broth, broth without peptone, Difco peptone, and Witte peptone

One-tenth ml of the toxin dilutions was injected into the thigh muscles of white mice weighing 20 g

DILUTIONS OF TOXIN 1556	SALINE	GUINEA PIG SERUM	SAVITA BROTH WITH 1% DIFCO PEPTONE	SAVITA BROTH WITHOUT PEPTONE	DIFCO PEPTONE 1%	WITTE PEPTONE 1%
1:2,000	3	3	—	3	1	2
1:4,000	0	3	2	3	2	2
1:8,000	0	3	2	3	2	2
1:16,000	0	3	3	3	2	3
1:32,000	—	3	4	3	5	L.T. 2
1:64,000	—	3	4	L.T. 1	5	L.T. 2
1:128,000	—	3	L.T. 2	L.T. 3	L.T. 1	L.T. 2
1:256,000	—	G.T. 4	L.T. 2	L.T. 3	L.T. 2	L.T. 2
1:512,000	—	—	0	—	—	—

Numerals indicate day of death.
G.T. = General tetanus, numerals indicating day of onset.
L.T. = Local tetanus, numerals indicating day of onset.
0 = No symptoms.
— = Not done.

broth, whereas Witte peptone has a weaker effect, but the potentiating effect is not exclusively due to the peptone content. Savita broth without peptone also potentiates markedly. Whether this effect is due to a single substance or to a variety of substances is still undecided.

The following experiments were likewise conducted with toxin 1556, but the broth was examined undiluted and in the dilutions 1:10, 1:100, and 1:1,000. As may be seen from table 2, the potentiating effect of a broth dilution of 1:10 is just as strong as the effect of undiluted broth. A dilution of 1:100 diminishes the effect by only one-half. But even in a dilution of 1:1,000 broth still has a marked potentiating effect. The significance of these results for the titration of tetanus toxins will be discussed below.

Differences in the potentiability of tetanus toxins. Differences in the potentia-

bility of tetanus toxins were uncovered by the following experiment: We tried to examine the absorption of tetanus toxin from the intraventricular fluid into the general circulation. Twenty lethal doses of toxins 1175 H and 103 were injected intracerebrally in guinea pigs weighing 250 g, and after various intervals samples

TABLE 2

Potentiation of tetanus toxin by various dilutions of broth

One-tenth ml of the toxin dilutions was injected into the thigh muscles of white mice weighing 20 g

DILUTIONS OF TOXIN 1556	SALINE	BROTH UNDILUTED	BROTH 1:10	BROTH 1:100	BROTH 1:1,000
1:500	2	—	—	—	—
1:1,000	7	—	—	—	—
1:2,000	L.T. 3	—	—	—	6
1:4,000	0	—	—	1	6
1:8,000	—	—	1	2	L.T. 1
1:16,000	—	5	2	4	L.T. 1
1:32,000	—	5	4	L.T. 1	—
1:64,000	—	L.T. 3	L.T. 1	L.T. 1	—
1:128,000	—	L.T. 3	L.T. 1	L.T. 1	—

Numerals indicate day of death.
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0 = No symptoms.
— = Not done.

TABLE 3

Difference in potentiability of various tetanus toxins by guinea pig serum

One-tenth ml of the toxin dilutions was injected intramuscularly in white mice weighing 20 g

TOXIN DILUTIONS	TOXIN 103		TOXIN DILUTIONS	TOXIN 1175 H	
	Saline	Serum		Saline	Serum
1:500	5	—	1:1,600	4	2
1:1,000	6	5	1:3,200	L.T. 2	3
1:2,000	8	5	1:6,400	0	4
1:4,000	0	0	1:12,800	0	4
1:8,000	0	0	1:25,600	0	9
1:16,000	0	0	1:51,200	0	L.T. 2
1:32,000	0	0	1:102,400	—	L.T. 2

Numerals indicate day of death.
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0 = No symptoms.
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of blood were taken and titrated for the presence of tetanus toxins in white mice weighing 20 g. The total amount of toxin 103 recovered from the blood was slightly less than the amount injected. In the experiment with toxin 1175 H, however, apparently much more toxin was recovered from the blood than had

been injected. Obviously this result could only be due to the potentiation of the toxin by the serum of the animal. We, therefore, carried out potentiation experiments with the two toxins.

TABLE 4

Potentiation of 31 tetanus toxins by guinea pig serum or broth

TOXIN	0.1 ML	TOXIN	0.1 ML	TOXIN	0.1 ML
G	1:50 1:50	641B	1:800 1:12,800	1346	1:1,000 1:4,000
8	1:100 1:200	5	1:800 1:6,400	T	1:1,600 1:25,600
3	1:100 1:400	15	1:800 1:1,600	K	1:1,600 1:12,800
2	1:200 1:1,600	3	1:800 1:3,200	D	1:1,600 1:12,800
1375	1:400 1:800	6	1:800 1:3,200	E	Z:1,600 1:12,800
22	1:400 1:400	A	1:800 1:6,400	F	1:1,600 1:12,800
H	1:400 1:6,400	B	1:800 1:25,600	641B	1:1,600 1:12,800
4	1:400 1:25,600	C	1:800 1:12,800	L	1:1,600 1:6,400
7	1:400 1:25,600	103	1:800 1:800	103	1:2,000 1:2,000
1346	1:500 1:16,000	103	1:1,000 1:1,000	1346	1:2,000 1:4,000
				1175H	1:4,000 1:128,000

One-tenth ml of each dilution of toxin was injected intramuscularly in white mice weighing 20 g. For each toxin the first row gives the lethal dose in saline. The second row gives the lethal dose in broth or serum. The lethal doses of toxins 3, 103, and 1346 were determined at various intervals after their preparation.

As may be seen from table 3, toxin 1175 H is strongly potentiated but toxin 103 is not potentiated at all. In the course of 2 years we repeated this experiment several times. Invariably toxin 103 was not potentiated either by broth or by serum.

In table 4 are recorded the results of potentiation experiments with a considerable number of tetanus toxins. It will be seen that there were 3 toxins (103, G,

and 22) which were not potentiated at all and others, like toxins 8, 1375, 15, and 1346, which were only very slightly potentiated. Most of the toxins, however, were strongly potentiated.

The question presented itself whether these results are indicative of qualitative differences between the individual toxins or whether they are simply related to the strength of the toxins. It must be kept in mind that the lethal doses of weak toxins contain a considerable amount of broth, whereas very little broth is present in the lethal doses of strong toxins. Weak toxins, therefore, may fail to be potentiated because they are already more or less potentiated by the broth contents of their lethal doses. The results recorded in table 3 lend no support to this explanation. Toxin 103 was in the beginning one of the strongest toxins at our disposal. Moreover, toxins 1375, 22, H, 4, and 7 all had lethal doses of 0.1 ml of a 1:400 dilution. The potentiation effect, however, varied from 1:1 for toxin 22 to 64:1 for toxin 7. The differences in potentiability, therefore, indicate

TABLE 5

Potentiation of toxin 1556 by broth in rabbits weighing 2,500 g

TOXIN 1556	DILUTIONS OF TOXIN MADE IN SALINE	DILUTIONS OF TOXIN MADE IN BROTH
0.1 ml undiluted	1, 4	4
" " 1:2.5	8, 9	4
" " 1:5	10, 16	2
" " 1:10	8, L.T. 3	5
" " 1:20	L.T. 3, —	G.T. 5
" " 1:40	—, —	0

Numerals indicate day of death.
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qualitative differences between the individual toxins. It may be remembered that other qualitative differences between tetanus toxins have been described in previous communications. Tetanus toxins were found to differ in the ratios of the lethal doses for the rabbit and the guinea pig (Friedemann, Zuger, and Hollander, 1939; Smith, 1943; Friedemann and Hollander, 1943) and in their avidities for nerve tissue (Friedemann and Hollander, 1943; Friedemann, Hollander, and Traub, 1946). It is unknown at the present time whether these various manifestations of qualitative differences are interrelated or independent of each other.

The potentiation effect in different animal species. The experiments reported in the preceding sections were conducted on mice. The results are approximately the same in guinea pigs. It will be seen from table 5 that the potentiation of toxin 1556, which was very strong in mice and guinea pigs, is negligible in rabbits. We further observed no appreciable potentiation in cats. It is questionable, however, whether this result indicates fundamental differences between animal

species. Both the rabbit and the cat are relatively resistant to tetanus toxin. The question, therefore, arises whether or not the broth content of the lethal dose is sufficient to produce maximal potentiation. This question will be answered only when media are available which contain no potentiating substances or when it is possible to conduct these experiments with purified toxins.

Some investigations on the mechanism of the potentiation effect. It may be questioned whether the term "potentiation effect" properly describes the observations reported in the preceding sections. According to Bronfenbrenner (1924), broth and serum act as buffers that prevent the spontaneous deterioration of botulinus toxin in saline. Halter (1936) and Neter (1942) have advanced the same explanation for the effect of broth and peptones on tetanus toxin. According to these authors, therefore, titrations of botulinus and tetanus toxins in broth, peptone, or serum would give correct results, but titrations in saline would be at

TABLE 6

Influence on toxicity of the time interval between preparation of dilutions of toxin and their injection

TOXIN DILUTIONS	A	B
1:500	1	1
1:1,000	1	2
1:2,000	2	3
1:4,000	L.T. 4	4

In the experiment recorded in column *A* the dilutions of toxin 1556 were injected immediately after their preparation. In the experiments recorded in column *B* the dilutions were kept at room temperature for 40 minutes before injection. All dilutions were made in saline, and 0.1 ml of each dilution was injected intramuscularly in white mice weighing 20 g.

Numerals indicate day of death.

L.T. = Local tetanus, numerals indicating day of onset.

fault. If this explanation be accepted, it is obviously unjustifiable to use the term "potentiation" in connection with our observations.

In the following experiments evidence will be presented to the effect that our observations can be explained only by a real potentiation. In the first place, the dilutions of tetanus toxin in saline were injected immediately after their preparation. It is highly improbable that in the few minutes, at most, elapsing between the preparation of the dilutions and their injection enough toxin would be destroyed to explain our results. Furthermore, if the toxin deteriorated rapidly in saline, it would be inexplicable that, in the rabbit, dilutions of toxin in saline and broth have the same potency. The experiment recorded in table 6 shows that even if the dilutions of toxin are kept at room temperature for 40 minutes no loss of toxicity is observed.

To exclude the very unlikely possibility that the toxin might be destroyed immediately upon its dilution in saline, toxin 1175 H was first diluted in saline 1:10, 1:100, and 1:1,000, and further dilutions were made in serum. As a con-

trol, dilutions of the toxin were made in saline and in serum from the outset. As may be seen from table 7, the titer is almost exactly the same irrespective of whether the toxin is diluted in broth from the outset or whether it is first diluted in saline 1:10, 1:100, or 1:1,000 and then broth added. This experiment shows conclusively that no toxin is destroyed immediately upon dilution in saline.

The same conclusion follows from neutralization experiments with antitoxin. If $\frac{31}{32}$ of the toxin were destroyed in saline, there should be a considerable difference in the antitoxin requirements for toxin diluted in saline or broth. We have shown in a previous communication (Zuger, Hollander, and Friedemann, 1939), and have confirmed the result time and again, that neither broth nor serum has any influence on the neutralization of tetanus toxin by antitoxin. There is,

TABLE 7

Potentiation of tetanus toxin by guinea pig serum after preliminary dilutions in saline

TOXIN DILUTIONS	A	B	C	D	E
1:2,000	2	—	1	1	2
1:4,000	3	—	2	2	2
1:8,000	L.T. 3	—	2	2	2
1:16,000	0	2	2	2	3
1:32,000	—	4	3	3	5
1:64,000	—	7	4	6	5
1:128,000	—	8	6	6	5
1:256,000	—	L.T. 2	L.T. 2	6	L.T. 2
1:512,000	—	L.T. 3	L.T. 3	0	L.T. 3
1:1,024,000	—	0	0	0	0

In column *A* all dilutions of toxin 1175 H were made in saline. In column *B* all dilutions were made in guinea pig serum. In columns *C*, *D*, and *E* preliminary dilutions of 1:10, 1:100, and 1:1,000, respectively, were made in saline, and all further dilutions were made in guinea pig serum.

One-tenth ml of each dilution was injected intramuscularly in white mice weighing 20 g. Numerals indicate day of death.

L.T. = Local tetanus, numerals indicating day of onset.

0 = No symptoms.

— = Not done.

therefore, abundant evidence that the effect of broth and serum on tetanus toxin is a real potentiation phenomenon.

We have conducted a large number of experiments in an attempt to elucidate the mechanism of this potentiation phenomenon. Since botulinus and tetanus toxins are potentiated but diphtheria toxin is not (Zuger, Hollander, and Friedeman, 1939), it was felt that this phenomenon might have something to do with the neurotropic character of the toxins. We thought of the possibility that potentiating substances might promote the entry of the toxins into the nerve endings. Our experiments in that direction, however, were inconclusive. We further investigated the influence of potentiating substances on neurotropic viruses. The potency of intramuscularly injected rabies virus in mice, however, was not enhanced by any of the potentiating substances. At the present time we

are not in position to offer a satisfactory explanation of the potentiation phenomenon.

SUMMARY AND DISCUSSION

In the preceding sections evidence of the potentiation of tetanus toxin by broth and serum has been presented. The most important results may be summarized as follows:

(1) Broth, even in a dilution of 1:1,000, has a marked potentiation effect on some tetanus toxins.

(2) Some tetanus toxins are strongly potentiated by broth and serum, but other toxins are not potentiated at all. The potentiation phenomenon, therefore, reveals qualitative differences between tetanus toxins.

(3) The potentiation phenomenon is marked in mice and guinea pigs, but absent in cats and rabbits.

(4) Broth and serum do not act as buffers which prevent a deterioration of the toxin in saline. The effect of broth and serum on tetanus toxin is a real potentiation phenomenon.

Since it is a widely accepted opinion that tetanus toxin deteriorates rapidly in saline, it has been recommended that broth or peptone be added to the dilutions of toxin in order to prevent the latter's destruction. Our experiments show that this procedure introduces a serious source of error. The addition of broth or peptone enhances very considerably the potency of some tetanus toxins, but it leaves the potency of others unaffected. This method, therefore, gives not only too high values for potentiable toxins but may lead to an entirely erroneous estimation of the relative potencies of toxins.

Our experiments further stress the necessity of distinguishing between toxins and toxic filtrates. The latter contain substances which have a very marked effect on the potency of the toxins. We have seen that broth even in a dilution of 1:1,000 still has a marked potentiating effect. A correct and reliable determination of the potency of toxins, therefore, will be possible only under one of the following conditions: (1) that toxins are produced in media which contain no potentiating substances; (2) that the titrations are made with purified toxins; or (3) that only highly potent toxins are used for which the effect of the broth content on the titer is negligible. Of these procedures only the last one is practicable. Actually, the National Institute of Health, although for other reasons, prescribes that, for the production of toxoids, only toxins containing at least 10,000 minimal lethal doses per ml should be used. In research work, however, weaker toxins will sometimes be used, and it will be necessary to have in mind the fallacies which the use of such toxins may involve.

Another pertinent subject is the titration of tetanus toxin in blood. Ramon and Descombey (1931) studied the absorption of tetanus toxin from the intraventricular fluid into the blood. Abel, Evans, and Hampil (1936) investigated in a similar way the absorption of the toxin from muscle. A number of investigators (Knorr, 1898; Marie, 1897; Blumenthal, 1898) injected tetanus toxin intravenously and followed up its persistence in the blood.

The experiment reported in table 3 (also see text) shows that the results are very markedly determined by the potentiability of the toxins used in these experiments. The apparent titers in the blood may be from 16 to 64 times too high in experiments with potentiable toxins.

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